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To be submitted to Laboratory Investigation, January 1992.
This is an introductory paper to a series on vocal fold fibroblasts in culture which will include the effects of laser irradiation on this cell type. This work was supported in part from a grant from ONR.

Title: Canine Vocal Fold Fibroblasts in Culture: Expression of α-Smooth Muscle Actin and Modulation of Elastin Synthesis.

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Running Title: Vocal Fold Fibroblasts

Abstract

Fibroblasts from canine vocal fold tissue have been isolated and maintained in culture. These cells express vimentin and α-smooth muscle actin, the characteristic marker of myofibroblast differentiation. Elastin production by vocal fold fibroblasts was compared to that of skin fibroblasts. The effects of transforming growth factor-β and hydrocortisone on elastin production were examined. Vocal fold fibroblasts in culture have a high elastogenic capacity compared to skin fibroblasts, and their elastin production can be enhanced by hydrocortisone (1.3μM) and TGF-β (10ng/ml). We conclude that the cells within the vocal fold are myofibroblastic in nature, and have high elastogenic potential *in vitro*. We suggest that connective tissue remodelling may be a continuous process in the vocal fold.

Key words: vocal fold, myofibroblast, α-smooth muscle actin, elastin, TGF-β

Introduction

Impaired voice quality, due to trauma, surgery, or advancing age, is a serious problem for many individuals. The inability to communicate effectively can interfere with an individual's career, and can lead to serious psychological problems. The human vocal fold, viewed in cross-section, consists of an epithelial layer, three layers of connective tissue comprising the lamina propria, and the vocalis muscle (14). The connective tissue composition of the lamina propria determines the biomechanical properties of the vocal fold, and thus the range sounds which can be produced.

Alterations in voice quality, including changes in pitch and increased hoarseness, which occur with age (16), are known to be associated with structural changes. These changes occur mostly within the lamina propria and include decreased elastin content in the intermediate layer, and increased collagen content in the deep layer (12,16). Similar changes in collagen:elastin ratio have been observed in other tissues in response to various types of injury (3,18). Most elastin synthesis occurs during development (4), so elastin is not normally replaced after injury.

Vocal fold tissue is required to have higher elasticity than many other tissues in order to perform its normal function. Histological studies of vocal folds from a variety of mammals have shown that this tissue is rich in elastic fibers. This is particularly true for vocal folds in both human and dog which are highly vocalizing mammals (14). Clearly, replacement of the elastic tissue with collagen following injury will severely affect voice quality. As the vocal fold is a highly dynamic tissue and is in constant use, it might be reasonable to expect cells from within this tissue, in particular the fibroblasts within the lamina propria to be specialized in some way. Subclasses of fibroblasts, which are believed to be functionally differentiated, have been identified recently in both injured and normal lung (1).

This paper describes the isolation, culture and characterization of the vocal fold fibroblast. These studies demonstrate that vocal fold fibroblasts in culture express α-smooth muscle actin, which is characteristic of myofibroblasts. The results also show that these cells in culture have high elastogenic potential, and that their elastin synthesis can be increased by both transforming growth factor-beta (TGF-β) and hydrocortisone. The culture of fibroblasts from the vocal fold provides a model system in which to study the normal physiology and pathological changes leading to decreased voice quality which are currently poorly understood.

Experimental Design

Canine vocal fold explants were cultured using standard techniques. Primary cultures and cultures up to passage four were examined for the presence of the cytoskeletal components vimentin, α-smooth muscle actin and desmin, in order to establsh the phenotype of the vocal fold fibroblast. The production of elastin by vocal fold fibroblasts was measured by an indirect enzyme-linked immunosorbent assay, and was compared to elastin production by skin fibroblasts from the same species. The effects of TGF-β and hydrocortisone on elastin production were examined.

Results

Histology

The vocal fold is lined with stratified squamous epithelium, which is suitable for withstanding frictional stress. Nuclei of fibroblasts can be observed within the vocal fold mucosa, specifically within the loose connective tissue of the lamina propria (Figure 1a). There is an abundance of elastin within the vocal fold mucosa (Figure 1b) with both elastin and collagen dispersed throughout the canine vocal fold lamina propria (Figure 1c). The vocalis muscle is a striated muscle (Figure 1d).

Cultured Cells from the Vocal Fold Mucosa and Vocalis Muscle.

Cells from the vocal fold mucosa and vocalis muscle were isolated using standard explant culture techniques as described below. The small pieces of tissue were placed in a minimal amount of growth medium to encourage attachment to the petri dish. After 24 hours, the tissue was attached firmly enough so as to allow the gentle addition of growth medium to the cultures. The explants can be left in a small amount of growth medium for up to 72 hours before requiring additional medium.

Within two to four days, cells were observed migrating from the vocal fold mucosa explant. The first cells to migrate from the explant were epithelial-like cells as suggested by their cobblestone appearance (Figure 2). These cells migrate as a flat sheet of closely apposed cells, which do not overlap one another. One to two days later, fibroblasts begin to appear as single cells. These cells were recognized by their elongated appearance (Figure 3), and their migration out from the explant over the epithelial cells. The fibroblasts continued to migrate and eventually made up the bulk of the cell population in the culture dish. On very rare occasions, single fibroblast-like cells were observed migrating from the explants prior to the emergence of the epithelial layer. Subculture of the cells resulted in a pure population of fibroblasts as the epithelial cells remained attached to the culture dish following trypsinization. Although within the vocal fold mucosa these are small vessels which are composed of smooth muscle and endothelial cells, these were not evident in culture probably due to their low numbers compared to the abundance of fibroblasts.

Within 3-4 days muscle-like cells had migrated from the vocalis muscle explants (Figure 4). No epithelial monolayer was observed migrating from these explants.

Immunocytochemical Characterization.

Vocal fold fibroblasts which had migrated from explants and subcultured up to passage 4 expressed α–smooth muscle cell actin (Figure 5a). However, between passage 1 and passage 4, the percentage of cells which stained positively for this cytoskeletal protein decreased from approximately 90% in passage 1 cultures, to around 50% in cultures at passage 4. These cells also stained positively for vimentin (Figure 5b). Epithelial-like cells from the explant were negative both for α-smooth muscle cell actin and vimentin. Neither fibroblasts nor epithelial cells expressed desmin. Fibroblasts isolated from dog skin expressed vimentin but not α-smooth muscle actin.

Elastin Production.

Elastin production from canine vocal fold fibroblasts was compared to that of canine skin fibroblasts. Vocal fold fibroblasts produced approximately twice that of skin fibroblasts (Figure 6). Furthermore, elastin production by canine vocal fold fibroblasts was stimulated by both TGF-β (10ng/ml) and hydrocortisone (1.3μM) (Figure 7).

Discussion

The vocal fold is a unique mammalian tissue which exists in a highly dynamic environment. The human vocal fold differs structurally from that of the dog in that the lamina propria in humans is divided into three layers, while the dog has only two layers and, therefore, has no structure equivalent to the vocal ligament. In the human, the layers are clearly defined with most of the elastin present in the superficial and intermediate layers, while the deep layer consists mostly of collagen fibers. In contrast, the superficial layer of the canine lamina propria is dense with both collagen and elastin, while the deep layer is loose in fibrous components as observed by Hirano and Kurita (14) and confirmed in this study. However, owing to

the overall abundance of elastin within the tissue from both species, we considered the canine vocal fold to be a suitable model. In addition, the macroscopic structure of the canine larynx is very similar to that of the human larynx (14).

The abundance of elastin within vocal fold suggests that this tissue is specialized. Vocal fold fibroblasts can be obtained from the canine vocal fold and maintained in culture for several months without any difficulty. In this study we used fibroblast cultures no older than passage four (maximum of four weeks old) so as to keep as close to the normal phenotype as possible.

The phenotype of the vocal fold fibroblasts was determined by immunocytochemical analysis of the cytoskeletal components. Fibroblasts from the vocal fold express both vimentin and α-smooth muscle actin, but not desmin. Vimentin is expressed equally by cells from primary explants and passage four cultures. However, α-smooth muscle actin expression appears to diminish between passage one and passage four.

The expression of α-smooth muscle actin in fibroblasts was initially described as a characteristic of the "injury" phenotype, and was observed in a population of cells which appeared transiently in experimental wound repair (5,8,26). Fibroblasts expressing α-smooth muscle actin have since been described in both injured and normal tissue (1,27). The population of cells sharing both fibroblast and smooth muscle properties are known as myofibroblasts. Several subclasses of myofibroblasts have now been identified and are distinguished by the pattern of staining for cytoskeletal components (27). The appearance of myofibroblasts in injury is thought to be central to the mechanisms of connective tissue remodelling and wound contraction (7), and may be important where structural integrity of the tissue is crucial for normal function.

Routine culture of fibroblasts on plastic and glass is reported to cause these cells to assume a phenotype closer to myofibroblasts (7). However, the fibroblasts

which migrated from the vocal fold explant expressed α-smooth muscle actin when examined after only six days in culture, and the expression of this cytoskeletal component appeared to diminish with time.

Vocal fold fibroblasts appear to be a myofibroblast-like cell expressing vimentin and α-smooth muscle actin. It is possible that the expression of α-smooth muscle actin in vocal fold fibroblasts reflects their normal function. Connective tissue remodelling may be a continuous process in the vocal fold. Changes in voice quality with age may be associated with loss of contractile properties and decreased elastin synthesis by vocal fold fibroblasts.

Early studies from our laboratory indicated that vocal fold-derived fibroblasts had a greater elastogenic capacity compared to skin-derived fibroblasts (11). The present study confirms that vocal fold fibroblasts produce approximately twice the elastin of skin fibroblasts *in vitro*. Vocal fold fibroblasts grown in culture appear to exhibit levels of elastogenesis which might represent their elastogenic potential *in vivo*. This might be useful for increasing elastin production in cells within the vocal fold which have undergone some kind of injury. Treatment with factors which stimulate elastin synthesis could restore elasticity to the tissue and thereby accelerate voice recovery after injury.

Candidates for stimulating elastin synthesis include glucocorticoids and TGF-β. The effects of glucocorticoids on connective tissue synthesis are usually inhibitory and include decreased synthesis of both collagen (22) and glycosaminoglycan (25). However, elastin synthesis in bovine nuchal ligament cells is increased by glucocorticoids (21). TGF-β is a known modulator of elastin synthesis and has been shown to upregulate elastin production in cultured porcine aortic smooth muscle cells (19) and granulation tissue fibroblasts (23). Our results show that treatment with TGF-β or hydrocortisone increased elastin production by vocal fold fibroblasts.

Mechanical deformation has been shown to increase elastin (24) and collagen synthesis (28) by aortic smooth muscle cells. Preliminary findings from our laboratory suggest that mechanical deformation of vocal fold fibroblasts stimulates elstin production (10). This could be useful in indicating whether light vocal fold exercise would be beneficial for voice recovery instead of complete voice rest which is now indicated after injury or surgery. The effects of mechanical deformation on elastin production by vocal fold fibroblasts will be the subject of a further study.

In summary, the vocal fold fibroblast appears to be a myofibroblast-like cell which has high elastogenic potential. The vocal fold may be a highly specialized tissue which, due to constant use, is in a continuous process of repair or remodelling in order to maintain normal function. Elastin production by the vocal fold fibroblast can be enhanced by TGF-β and hydrocortisone. This could be important in the treatment of injured or aged vocal folds for recovery of normal voice quality.

Methods

Histology. A whole larynx was obtained from a euthanized dog and was fixed in 10% formalin for 72 hours at 4°C. The vocal fold was removed and embedded in paraffin from which 4μm tissue sections were cut. The tissue was then stained with Masson's trichrome with Azan (M&A), Elastic van Gieson (EvG) or Hematoxylin and Eosin (H&E) using standard procedures.

Isolation and Culture of Vocal Fold Cells. Laryngeal tissue was obtained from dogs which had been euthanized by intravenous injection of a lethal dose of sodium pentobarbital. The tissue was collected in Hank's balanced salts solution buffered with 10mM HEPES (buffered-HBSS), containing penicillin (50U/ml), streptomycin (50μg/ml) and amphotericin B (12ng/ml). The tissue was rinsed twice with buffered-HBSS containing antibiotics, and placed in a petri dish where the larynx was cut open to expose the vocal folds. The vocal fold mucosa was elevated and a small strip of mucosa containing the epithelium and lamina propria, was removed using forceps and small scissors, and placed into a 15ml centrifuge tube. A small part of the vocalis muscle from deeper in the vocal fold was removed and placed into another 15ml centrifuge tube. The tubes were filled with buffered-HBSS containing antibiotics and the tissue washed and collected by centrifugation. Each tissue was then placed into a Petri dish and teased into small pieces (~1mm^3) using forceps and a scalpel. The pieces were collected and washed in buffered-HBSS by centrifugation, then resuspended in a small amount (3-5ml) of culture medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum, penicillin (50 U/ml), streptomycin (50μg/ml) and amphotericin B (12ng/ml) (DMEM+10%FCS). The suspension was poured into a 100 mm diameter petri dish (Falcon Labware) and the medium swirled so as to cover as much of the plate as possible. In some cases the explants were plated into Labtek™ 8-well slides (Nunc) for immunocytochemistry. The dishes were placed in an incubator (5% CO_2/95%

O_2) at 37°C. After 24 hours 10ml growth medium was added gently to the cultures. All tissue culture reagents were obtained from Gibco Biotechnologies, Grand Island, N.Y.

Isolation and Culture of Skin Fibroblasts. Skin tissue was obtained from the dorsal pelt of euthanized dogs and collected in buffered-HBSS containing antibiotics. The tissue was washed twice in buffered-HBSS then placed into a petri dish. The fascia was removed and small pieces of skin dissected from the pelt. The skin was teased into small (~1mm^3) pieces and cultured in the same manner as described for vocal fold tissue. All cell types were subcultured using standard trypsinization procedures.

Immunocytochemical characterization. Cells were subcultured onto Labtek™ 8-well slides and grown in DMEM+10%FCS. When the cells were 80-90% confluent, they were washed twice in buffered-HBSS then fixed in 10% formalin for 30 minutes and stored in Dulbecco's phosphate buffered saline (PBS) (Gibco) at 4°C in a humidified atmosphere. Prior to staining, the cells were permeabilized in acetone for 5 minutes at -20°C. The slides were placed in PBS for 2 minutes then the primary antibody was added in PBS +1% bovine serum albumin (BSA) at 1:50, 1:100 and 1:200 dilutions, and incubated for 2 hours. Antibodies used were monoclonal anti-α-smooth muscle actin antibody, monoclonal anti-desmin antibody and monoclonal anti-vimentin antibody. The slides were washed 3 times in PBS, then incubated with TRITC-conjugated goat anti mouse IgG for 1 hour. The slides were then washed 3 times in PBS, rinsed once in water, and mounted in glycerol:water (1:1). Photographs were taken using a Nikon Optiphot photomicroscope equipped with epiillumination and specific filters for rhodamine, and a plan apochromate X40/0.75 objective and Ektachrome 400 ASA color slide film (Eastman Kodak Co., Rochester, NY). All antibodies were obtained from Sigma Chemical Co., St. Louis, MO.

Elastin Synthesis Measurements. Measurements of elastin synthesis were obtained from canine vocal fold fibroblasts between passages 2 and 4, at the late log phase of

growth. Cells were subcultured onto 24-multi-well dishes (Costar, Cambridge, MA) and grown to confluence. The medium was then replaced with 1ml DMEM+10% Newborn calf serum (NBCS). This medium was used in order to maximize differences in elastin production in response to the treatments described, as elastin production by cells cultured in FCS is very high (unpublished observation). This medium was then collected at given time intervals and elastin production measured by an indirect enzyme-linked immunosorbent assay (9). Briefly, microtiter plates were coated with dog α-elastin (25ng) in Voller's buffer (30) and incubated at 4°C overnight. Competition plates containing standard antigen (bovine tropoelastin, Elastin Products Company, Owensville, MO) or samples and polyclonal rabbit anti-pig α-elastin (1:3000) (9) were incubated at 4°C overnight. The following day, the contents of the competition plate were transferred to the coated plate and allowed to react. The reaction was visualized using horseradish peroxidase-conjugated goat anti rabbit IgG (1:2000) (Kirkegaard and Perry Laboratories). The plates were read in a Thermomax plate reader (Molecular Devices Corporation) at OD490nm and the results extrapolated from a 4-parameter, non-linear regression analysis of the standard curve (24) using SOFTmax software (Molecular Devices). Results are expressed as molecular equivalents of soluble elastin normalized to cell number. Cell number was determined by DNA content as described previously (15).

Treatment with TGF-β and Hydrocortisone. TGF-β (provided by Dr. Rik Derynck, Genentech, Inc.) or hydrocortisone (Sigma Chem. Co.) at the given concentrations were added to late log phase cultures of vocal fold fibroblasts in DMEM + 10% NBCS for times indicated in the figures.

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Figure Legends

Figure 1(a). H&E stained canine vocal fold showing stratified squamous epithelial lining and nuclei of fibroblasts within the lamina propria (Xxx mag.)

Figure 1(b). Elastic van Gieson stained canine vocal fold showing disrtibution of elastin within the lamina propria (X200 mag.)

Figure 1(c). Masson's trichrome stained canine vocal fold showing distribution of collagen (X200 mag)

Figure 1(d). H&E stained canine vocal fold showing striated vocalis muscle (X200 mag.)

Figure 2. Canine vocal fold explant 4 days in culture showing epithelial monolayer (X250 mag.)

Figure 3. Canine vocal fold explant 6 days in culture showing fibroblasts (X250 mag.)

Figure 4. Canine vocalis muscle explant 4 days in culture showing muscle cells (X250 mag.)

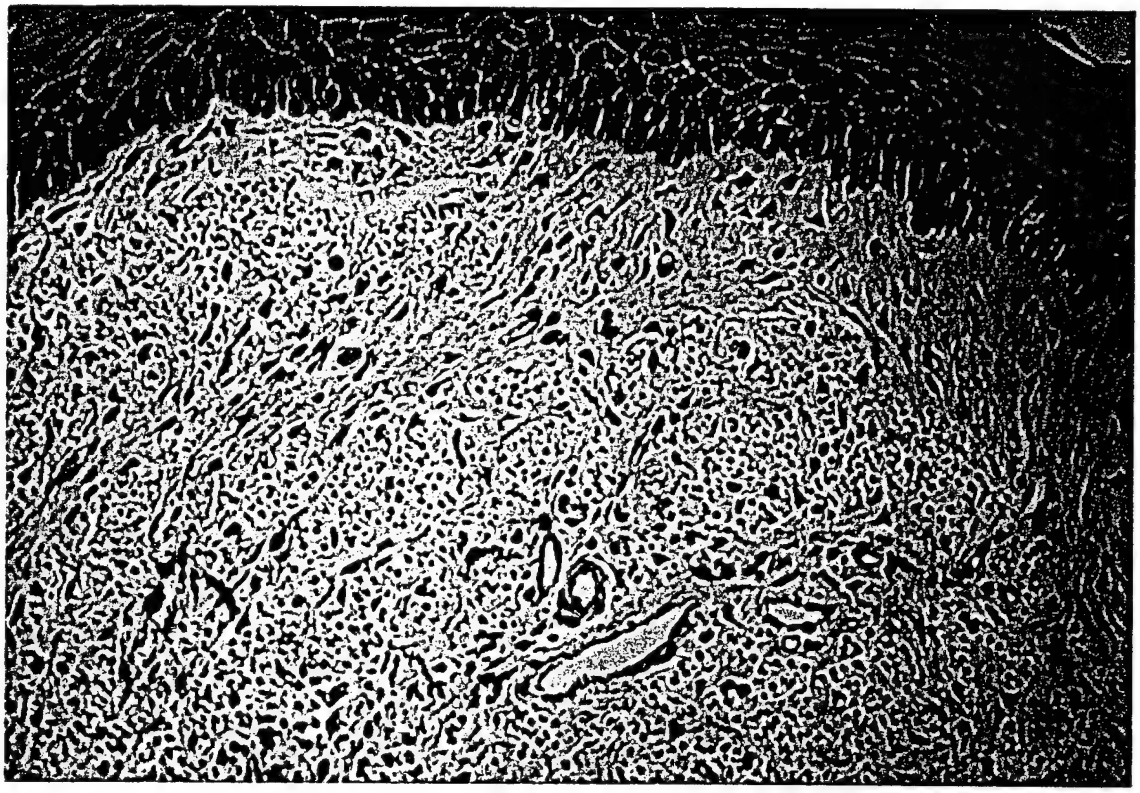
Figure 5(a). Vocal fold fibroblasts stained with antibody to a-smooth muscle actin (X300 mag.)

Figure 5(b). Vocal fold fibroblasts stained with anitbody to vimentin (X300 mag.)

Figure 6. Elastin production by canine skin vs canine vocal fold fibrobalsts. Each point represents the mean ± s.e. of two experiments performed in duplicate. $p<0.05$ using Student's paired t-test.

Figure 7. Effect of hydrocortisone and TGF-β on elastin production. Each point represents the mean ± s.e. of three experiments performed in duplicate. $p<0.05$ compared to control at each time point using Student's paired t-test.

Figure 1(a)



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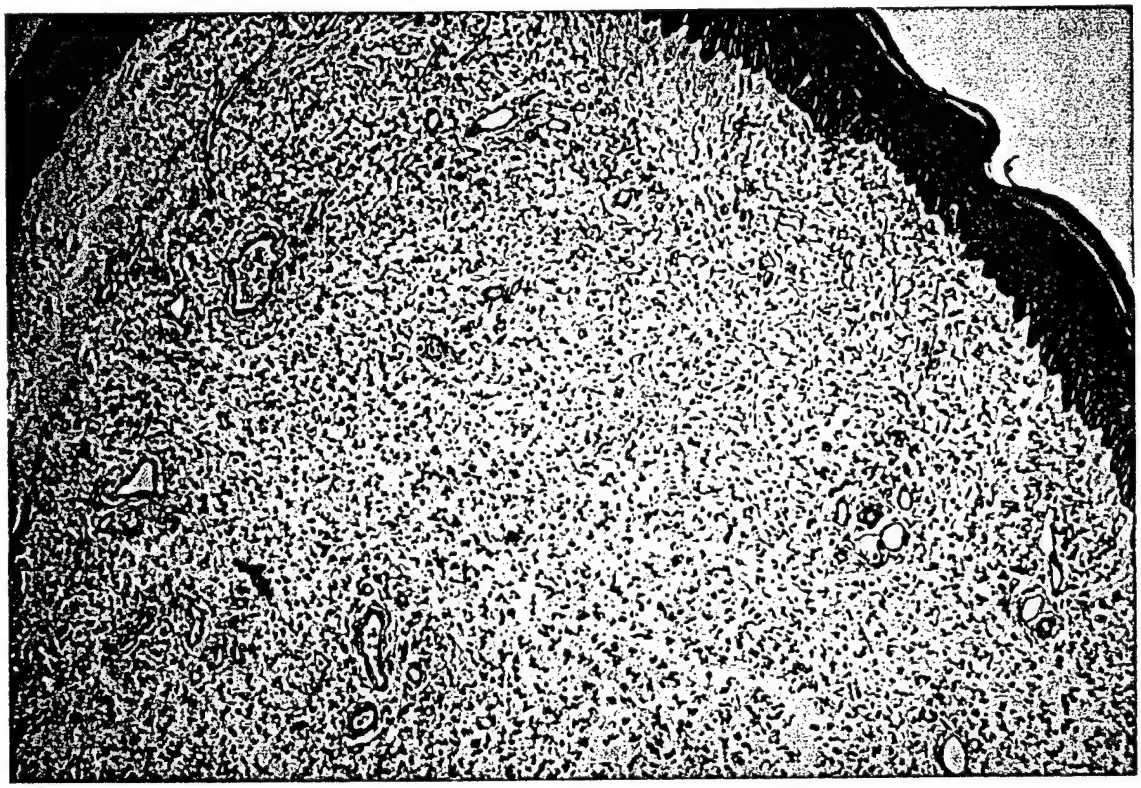

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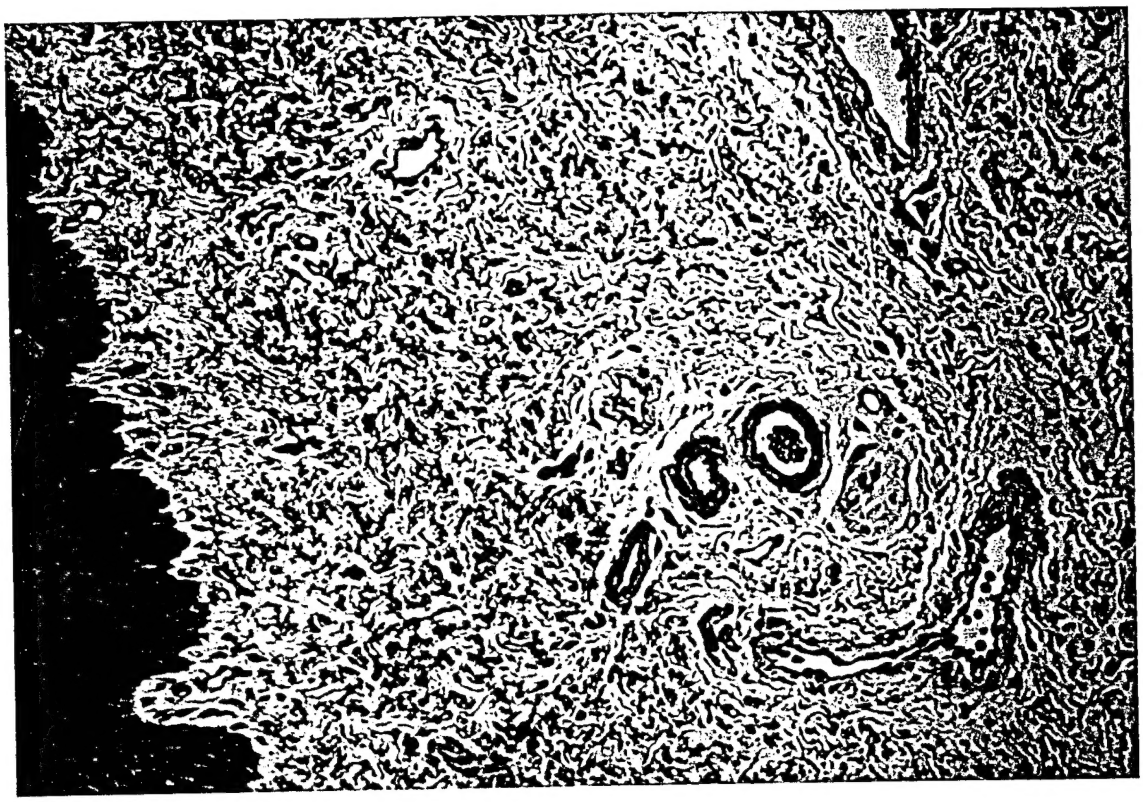
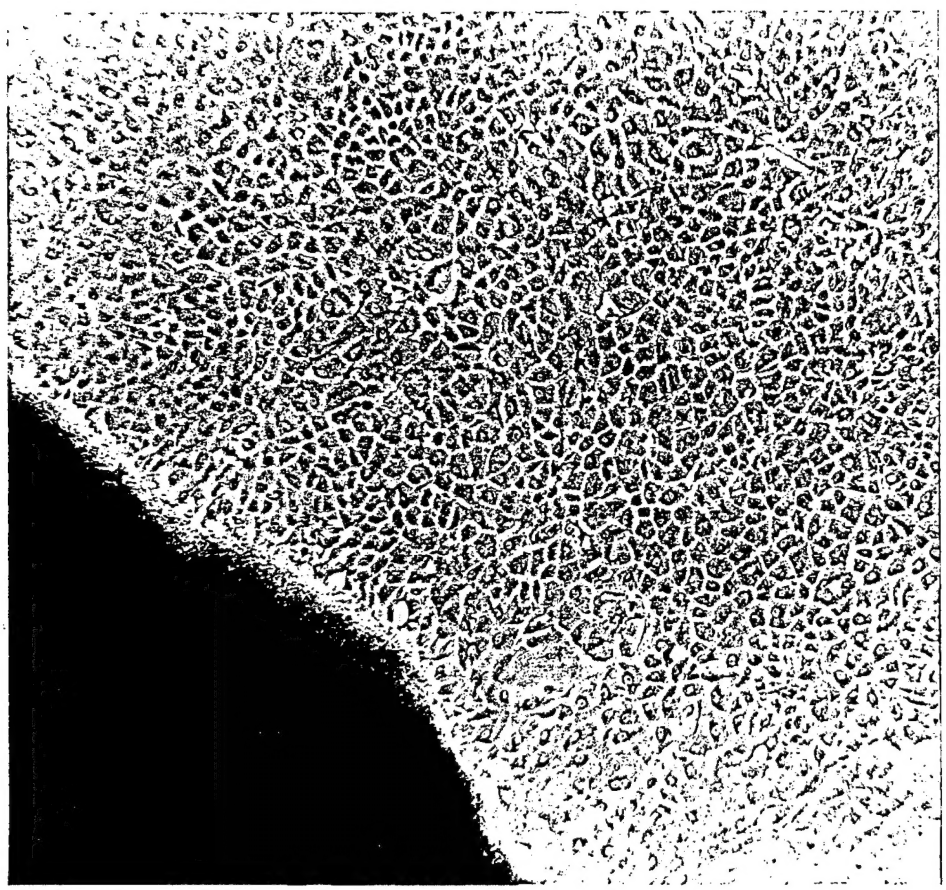


Figure 1(d)

Figure 2



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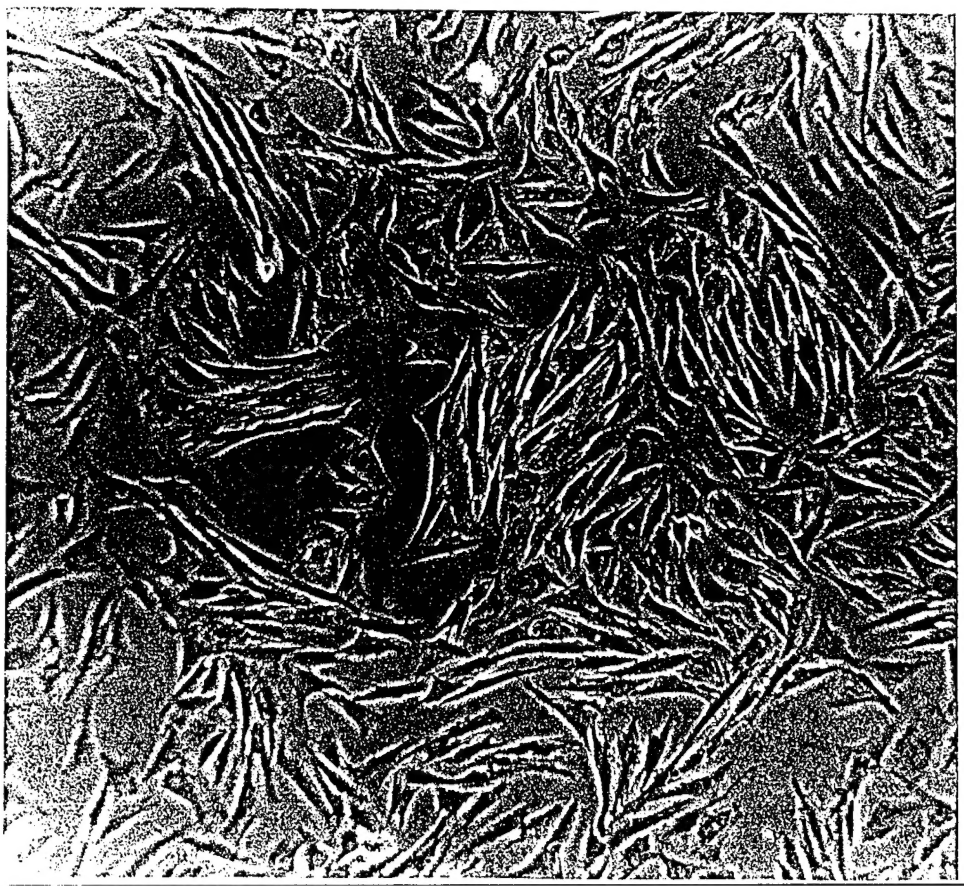

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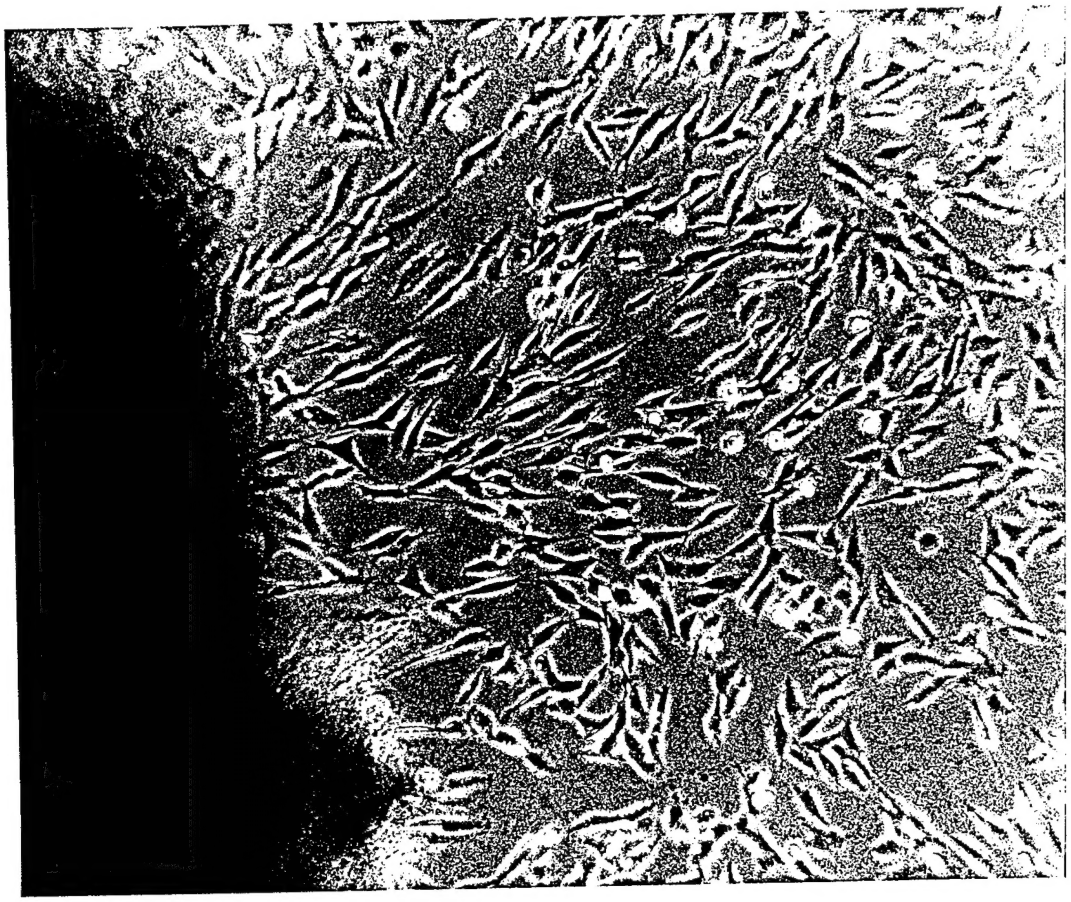

Figure 5

Figure 6

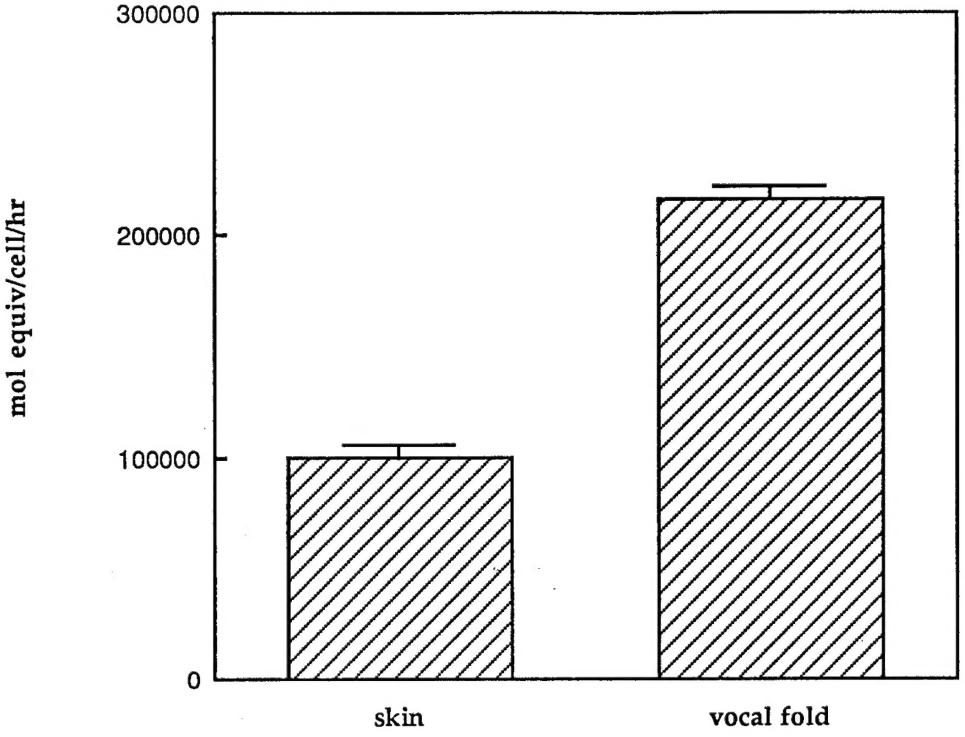

Figure 7